Differentiation of *Burkholderia* Species by PCR-Restriction Fragment Length Polymorphism Analysis of the 16S rRNA Gene and Application to Cystic Fibrosis Isolates

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Burkholderia cepacia*, which is an important pathogen in cystic fibrosis (CF) owing to the potential severity of the infections and the high transmissibility of some clones, has been recently shown to be a complex of five genomic groups, i.e., genomovars I, II (*B. multivorans*), III, and IV and *B. vietnamiensis*. *B. gladioli* is also involved, though rarely, in CF. Since standard laboratory procedures fail to provide an accurate identification of these organisms, we assessed the ability of restriction fragment length polymorphism (RFLP) analysis of amplified 16S ribosomal DNA (rDNA), with the combination of the patterns obtained with six endonucleases, to differentiate *Burkholderia* species. This method was applied to 16 type and reference strains of the genus *Burkholderia* and to 51 presumed *B. cepacia* clinical isolates, each representative of one clone previously determined by PCR ribotyping. The 12 *Burkholderia* type strains tested were differentiated, including *B. cepacia*, *B. multivorans*, *B. vietnamiensis*, and *B. gladioli*, but neither the genomovar I and III reference strains nor the genomovar IV reference strain and *B. pyrrocinia*[T] were distinguishable. CF clinical isolates were mainly distributed in RFLP group 2 (which includes *B. multivorans*[T]) and RFLP group 1 (which includes *B. cepacia* genomovar I and III reference strains, as well as nosocomial clinical isolates). Two of the five highly transmissible clones in French CF centers belonged to RFLP group 2, and three belonged to RFLP group 1. The remaining isolates either clustered with other *Burkholderia* species (*B. cepacia* genomovar IV or *B. pyrrocinia*, *B. vietnamiensis*, and *B. gladioli*) or harbored unique combinations of patterns. Thus, if further validated by hybridization studies, PCR-RFLP of 16S rDNA could be an interesting identification tool and contribute to a better evaluation of the respective clinical risks associated with each *Burkholderia* species or genomovar in patients with CF.

Burkholderia cepacia, first described as the agent of the onion soft rot (2), is an opportunistic human pathogen which may be responsible for various nosocomial infections and is especially problematic in patients with cystic fibrosis (CF) due to the potential severity of respiratory infections, the easy patient-to-patient spread of the organism, and its innate resistance to a wide range of antimicrobial agents (8). These data led to the introduction of various preventive guidelines in and outside hospitals (hygiene procedures, segregation of colonized patients, etc.) in order to reduce the risk of acquisition. Nevertheless, the clinical outcome in *B. cepacia*-infected CF patients is extremely variable, ranging from a fatal necrotizing pneumonia, the "cepacia syndrome" to an unmodified respiratory status (10). In the same way, large epidemics as well as small clusters of infection or unique colonizations have been reported on the basis of the genotypic analysis of isolates (17, 21). Owing to the diversity of clinical situations due to *B. cepacia*, the identification of pathogenicity and transmissibility markers is essential. The first requirement in achieving this goal is a precise bacteriological knowledge of the responsible organism which, as a matter of fact, proves difficult. First, the accurate identification of *B. cepacia* with widely used multitest commercial systems has been recently shown to be problematic (12). Second, the genus *Burkholderia* has undergone multiple changes since its individualization by Yabuuchi et al. in 1992 (28). Some species (formerly *B. pickettii* and *B. solanacearum*) have been transferred to another new genus, *Ralstonia* (29), whereas species either formerly belonging to the genus *Pseudomonas* (*B. cocovenenans* [30], *B. plantarii* and *B. glumae* [24], and *B. pyrrocinia* and *B. glathei* [25, 26]) or newly described (*B. vandii* [24], *B. vietnamiensis* [6], and *B. thailandensis* [1]) have been included in the genus *Burkholderia*. The involvement of these various species, most of which are phytopathogenic, as opportunistic agents in human pathology has not been assessed so far, whereas infections due to *B. gladioli* have already been reported in CF (4, 11), chronic granulomatous disease (20), and other immunocompromised states (9). Yet the bacteriological differentiation between *B. cepacia* and *B. gladioli* is not always clear-cut (23). Third, organisms presently identified as *B. cepacia* appear to be very heterogeneous and constitute a "complex" of phenotypically similar species among which five genomic groups, i.e., genomovars I, II, III, and IV and the formerly described *B. vietnamiensis*, could be differentiated on the basis of whole-cell protein analysis, nucleic acid studies, and cellular fatty acid composition; the individualization of genomovar II as a new species, *B. multivorans*, has been proposed as well (25). Moreover, the importance of genomovar III among CF isolates, especially epidemic clones, has been emphasized (8, 25).

These differentiation procedures of *Burkholderia* taxa are not appropriate for routine diagnosis, and the aim of the

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present study was to develop a molecular identification tool which could be accessible to clinical microbiology laboratories. Restriction fragment length polymorphism (RFLP) analysis of amplified 16S ribosomal DNA (rDNA), previously proven to be discriminant for the differentiation of fluorescent *Pseudomonas* species (14), was first applied to reference strains belonging to different species of the genus *Burkholderia*. Next, a large set of clinical isolates, each representative of a given clone previously determined by use of PCR ribotyping (5, 13, 22), was analyzed comparatively to type and reference strains so as to assess the distribution of the various *Burkholderia* species in CF and nosocomial infections in sporadic and epidemic situations. Furthermore, carbon substrate assimilation tests were performed on strains belonging to the different RFLP types in order to point out differential phenotypic characters.

MATERIALS AND METHODS

Bacterial strains. All of the strains studied are listed in Tables 1 and 2. Type strains were selected as the gold-standard representatives of each species; if not available, representative strains were chosen among those previously characterized by Vandamme et al. (25) as belonging to the different *B. cepacia* genomovars. The 18 type and reference strains analyzed (Table 1) were obtained from international culture collections and include species of the *B. cepacia* complex (25), i.e., genomovars I, II (*B. multivorans*), III, and IV and *B. vietnamiensis*; other *Burkholderia* species (*B. pyrrocinia*, *B. cocovenenans*, *B. gladioli*, *B. plantarii*, *B. caryophilli*, *B. andropogonis*, *B. glumae*, and *B. glathei*), and lastly two unrelated species (*Ralstonia pickettii* and *Stenotrophomonas maltophilia*). The 51 clinical isolates tested (Table 2), which were recovered from CF or non-CF patients, were selected on the basis of a previously performed genotypic analysis by PCR ribotyping among the French Observatoire cepacia collection and among strains kindly provided by N. Høiby (Copenhagen, Denmark), J. Govan (Edinburg, United Kingdom), K. Poole (Kingston, Ontario, Canada), and D. P. Speert (Vancouver, British Columbia, Canada). Each of these clinical isolates is representative of a different PCR ribotype (designated with capital letters).

All reference and clinical strains were analyzed with the API 20NE system (bioMérieux, Marcy-l'Etoile, France). Oxidase was checked by using dimethyl-paraphenylenediamine disks (bioMérieux). The results of the API NE biochemical tests and of the oxidase reaction were transformed into a numerical profile, further interpreted by using the API NE analytical profile index, which provided percentages of identification.

RFLP analysis of amplified 16S rDNA. The 18 reference strains and the 51 clinical isolates were tested. Furthermore, five isolates belonging to PCR ribotype F, four isolates belonging to PCR ribotype X, two isolates belonging to PCR ribotype D, eight isolates belonging to PCR ribotype A, two isolates belonging to PCR ribotype U, and two isolates belonging to PCR ribotype P were analyzed in order to check the homogeneity of the 16S rDNA restriction results within the same PCR ribotype.

DNA was prepared from chocolate agar (bioMérieux) cultures; a bacterial suspension, adjusted to 4 U of McFarland turbidity, was pelleted by centrifugation, washed, resuspended in 500 μl of distilled water, heated for 15 min at 95°C, and then centrifuged; the supernatant was stored at −20°C until used as a template.

Primers fD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTC AG-3') and rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3'), which are complementary to conserved regions of the 16S rDNA (27), were used in a 100-μl reaction mixture containing 200 μM deoxynucleoside triphosphates (Perkin-Elmer, Saint-Quentin en Yvelines, France), 0.1 μM concentrations of primers (Bioprobe Systems, Montreuil, France), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 2.5 U of *Taq* polymerase (Gibco-BRL Life Technologies, Gaithersburg, Md.), and 10 μl of template DNA or water for the negative control. An initial denaturing step of 95°C for 10 min was followed by 30 cycles of amplification (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) and a final extension step at 72°C for 10 min. DNA amplification was checked by electrophoresis of 5 μl of PCR product in a 1% agarose gel in Tris-borate-EDTA (TBE) buffer (pH 8.3) and by staining with ethidium bromide. Amplification products were stored at −20°C until digested. Restriction was performed separately with each enzyme by incubating 5 μl of amplified 16S rDNA overnight at the appropriate temperature with 5 U of endonuclease in a final volume of 25 μl. Six of the thirteen enzymes used by Laguerre et al. (14) to differentiate fluorescent *Pseudomonas* species were selected in our study because of their discriminatory power on *Burkholderia* species based on published rDNA sequences, i.e., *Alu*I, *Cfo*I, *Dde*I, *Msp*I, and *Nci*I (Gibco-BRL) and *Bss*KI (i.e., *Scr*FI) (New England Biolabs, Beverly, Mass.). The restriction fragments were separated by electrophoresis in 3% agarose (Nusieve GTG; FMC, Rockland, Maine) in TBE buffer and stained with ethidium bromide by using marker VIII (Boehringer Mannheim, Meylan, France) as a molecular size ladder. The profiles obtained

with each endonuclease were designated with uppercase letters. RFLP groups, designated with arabic numbers, were defined by the combination of the restriction profiles obtained with four of the six enzymes, i.e., *Alu*I, *Cfo*I, *Dde*I, and *Msp*I, and RFLP subgroups, designated with lowercase letters, were further defined on the basis of *Nci*I and *Bss*KI patterns (Tables 1 and 2).

Carbon substrate assimilation tests. A complete assimilation study (carbohydrates, amino acids, and organic acids) was performed on the 18 reference strains and on 35 clinical isolates belonging to 35 different PCR ribotypes and 14 16S rDNA genotypes by using Biotype 100 strips inoculated with bacterial suspensions in Biotype medium 1 according to the instructions of the manufacturer (bioMérieux). The strips were incubated at 30°C for 4 days, and growth was recorded at days 2 and 4. Assimilation data were entered in an Apple Macintosh computer with the Taxotron software (Institut Pasteur, Paris, France).

RESULTS

API 20NE identification. The results obtained with reference strains are given in Table 1. *B. cepacia*, *R. pickettii*, and *S. maltophilia* are the only species among those tested which are included in the API 20NE database. Among the seven strains belonging to the *cepacia* complex, *B. cepacia* (ATCC 25416[T]) and *B. cepacia* genomovar II (*B. multivorans* LMG 13010[T]) were identified as *B. cepacia*, one strain (ATCC 25609) could not be identified, and the identification was incomplete for the four remaining strains (*B. cepacia* was not differentiated from *P. aureofaciens*). The nine type strains belonging to other *Burkholderia* species were either misidentified (five strains) as *B. cepacia* or as *B. cepacia* or *P. aureofaciens*, not identified (three strains [*B. cocovenenans*, *B. caryophilli*, and *B. andropogonis*]), or not typeable (one strain [*B. glathei*]). Finally, the type strains of *R. pickettii* and *S. maltophilia* were identified with a percentage of identification of >95%.

Among the 51 clinical isolates tested (Table 2), 24 (47%) were identified as *B. cepacia* with a percentage of identification of >99%, 15 (28%) were incompletely identified (*B. cepacia*, *P. aureofaciens*, or *P. fluorescens*), 1 (2%) was identified as *Aeromonas salmonicida* because of negative assimilation tests, and 11 (21%) were not identified. However, 6 of the 11 unidentified isolates, which were found to be oxidase negative, would be identified as *B. cepacia* with a percentage of identification of >99% if considered oxidase positive.

16S rDNA RFLP profiles. The representatives of the five species or genomovars of the *B. cepacia* complex were classified in four RFLP groups (Table 1). *B. cepacia* genomovar I and III strains belonged to the same RFLP group (group 1), whereas *B. cepacia* genomovar II (*B. multivorans*), *B. cepacia* genomovar IV, and *B. vietnamiensis* belonged to different RFLP groups (groups 2, 3, and 4, respectively).

The nine type strains representing other *Burkholderia* species were classified in eight RFLP groups. *B. pyrrocinia* harbors the same restriction patterns as *B. cepacia* genomovar IV, whereas other species are easily differentiated from the *B. cepacia* complex. Interestingly, *B. cocovenenans* and *B. gladioli* pathovar gladioli belong to the same RFLP group, being only differentiated by their *Bss*KI profile, and the two pathovars of *B. gladioli* could be differentiated on the basis of their *Dde*I profile. The 16S rDNA genotypes of *R. pickettii* and *S. maltophilia* type strains were clearly different from those of *Burkholderia* strains. Figure 1 illustrates the RFLP profiles obtained with *Dde*I for the different genomovars and species studied.

The analysis of several clinical isolates belonging to the same PCR ribotype confirmed the homogeneity of the 16S rDNA restriction results, thus validating the selection of one isolate per PCR ribotype. The 51 representative CF and non-CF clinical isolates (Table 2) were classified into nine RFLP groups: 1, 2, 3, 4, 5, 6, 14, 15, and 16. The majority of the strains (43 of 51 [83%]) was shown to belong to 2 of the 9 groups defined. (i) Group 2 (26 of 51 strains [51%]) is characterized by an "I"

TABLE 1. 16S rDNA RFLP profiles of the 18 reference strains analyzed in this study

Taxon[a]	Strain[b]	Other designation[b]	Source of isolation	API 20NE numerical profile, taxon (% identification)	AluI	CfoI	DdeI	MspI	NciI	BssKI	RFLP[c] Group	RFLP[c] Subgroup
Cepacia complex												
B. cepacia genomovar I	ATCC 25416[T]	LMG 1222[T]	*Allium cepa* L.	1 477 577, *B. cepacia* (99.9)	A	A	A	A	AH	AH	1	1a
B. cepacia genomovar I	ATCC 17759	LMG 2161	Forest soil (Trinidad)	0 067 577, *B. cepacia* (95.5), *P. aureofaciens* (4.4)	A	A	A	A	A	A	1	1b
B. cepacia genomovar I	ATCC 25609	LMG 6981	Human (bronchial washings)	1 477 576, unidentified	A	A	A	A	A	A	1	1b
B. multivorans genomovar II	LMG 13010[T]	NCTC 13007[T]	Human (CF patient, Belgium)	1 477 577, *B. cepacia* (99.9)	A	A	I	A	AI	AI	2	2a
B. cepacia genomovar III	LMG 12615	NCTC 13008	Human (CF patient, UK)	0 067 577, *B. cepacia* (95.5), *P. aureofaciens* (4.4)	A	A	A	A	A	A	1	1b
B. cepacia genomovar IV	LMG 14294	NCTC 13011	Human (CF patient, Belgium)	0 057 577, *B. cepacia* (50.3), *P. aureofaciens* (48.9)	A	B	B	A	H	H	3	
B. vietnamiensis	TVV75[T]	LMG 10929[T]	Rice rhizosphere (Vietnam)	1 067 577, *B. cepacia* (93.3), *P. aureofaciens* (6.7)	A	A	J	A	A	A	4	
Genus *Burkholderia* (other species)												
B. pyrrocinia	LMG 14191[T]	ATCC 15958[T]	Soil	0 067 577, *B. cepacia* (95.5), *P. aureofaciens* (4.4)	A	A	A	B	B	A	3	
B. cocovenenans	LMG 11626[T]	ATCC 33664[T]	Poisoned bongkrek (Java)	1 047 555, unidentified	B	B	A	B	A	F	5	5b
B. gladioli pathovar *gladioli*	CFBP 2427[T]	ATCC 10248[T]	*Gladiolus* sp.	1 047 577, *B. cepacia* (68.5), *P. aureofaciens* (28.7), *P. fluorescens* (2.8)	B	B	B	B	A	A	5	5a
B. gladioli pathovar *alliicola*	CFBP 2422[T]	ATCC 19302[T]	*Allium cepa* L. (United States)	1 057 577, *B. cepacia* (95.5), *P. aureofaciens* (4.4)	B	B	F	B	H	H	6	
B. plantarii	LMG 9035[T]		*Oryza sativa* L. (Japan)	1 077 577, *P. aureofaciens* (59.9), *B. cepacia* (39.5)	B	B	G	B	A	A	7	
B. caryophylli	LMG 2155[T]	ATCC 25418[T]	*Dianthus caryophyllus* L.	1 047 544, unidentified	B	B	A	B	B	B	8	
B. andropogonis	LMG 2129[T]	ATCC 23061[T]	*Holcus sorghum* L. (United States)	1 047 044, unidentified	C	C	C	C	A	C	9	
B. glumae	CFBP 2430[T]	NCPPB 2391[T]	*Oryza sativa* (Japan)	No growth	A	B	L	F	A	J	10	
B. glathei	LMG 14190[T]	ATCC 29195[T]	Soil (Germany)	5 077 557, *B. cepacia* (98.4)	G	G	H	B	I	A	11	
Other genera												
R. pickettii	ATCC 27511[T]		Human (tracheotomy)	1 241 455, *R. pickettii* (95.2)	D	D	D	D	D	D	12	
S. maltophilia	ATCC 13637[T]		Human (oropharynx)	1 472 341, *S. maltophilia* (99.9)	E	E	E	E	E	E	13	

PCR ribo-type	No. of patients; geographic location; source[a]	Isolate tested (reference)	API 20NE numerical profile, taxon (% identification)	AluI	CfoI	DdeI	MspI	NciI	BssKI	Group	Subgroup
F	32 CF patients; France (Giens, 30; Dijon, 1; Pessac 1); sputum, blood culture	2.31	1 447 577, *B. cepacia* (99.9)	A	A	I	A	A	AI	2	2a
X	22 CF patients; France (Paris, 21; Toulouse, 1); sputum	1.55	5 466 577, *B. cepacia* (99.9)	A	A	I	A	A	AI	2	2a
BN	1 CF patient; France (Tours); sputum	3.75	5 067 577, *B. cepacia* (99.9)	A	A	I	A	A	AI	2	2a
BF	1 CF patient; France (St. Nazaire); sputum	3.6	1 067 577, *B. cepacia* (93.3), *P. aureofaciens* (6.7)	A	A	I	A	A	AI	2	2a
BO	1 CF patient; France (Lille); sputum	3.79	0 047 577, *B. cepacia* (75.4), *P. aureofaciens* (20.3), *P. fluorescens* (4.3)	A	A	I	A	A	AI	2	2a
K	2 CF siblings; France (Rouen); sputum	3.67	1 047 577, *B. cepacia* (68.5), *P. aureofaciens* (28.7), *P. fluorescens* (2.8)	A	A	I	A	A	AI	2	2a
AY	1 CF patient; France (Rouen); sputum	2.48	5 067 577, *B. cepacia* (99.9)	A	A	I	A	A	AI	2	2a
I	1 CF patient; France (Giens); sputum	1.20	1 467 577, *B. cepacia* (99.9)	A	A	I	A	A	AI	2	2a
AV	1 CF patient; Denmark (Copenhagen)	A47	1 447 577, *B. cepacia* (99.9)	A	A	I	A	A	AI	2	2a
AO	1 CF patient; Denmark (Copenhagen)	A40	5 467 573, unidentified[b]	A	A	I	A	A	AI	2	2a
AQ	1 CF patient; Denmark (Copenhagen)	A42	5 010 004, *A. salmonicida* (90.2)	A	A	I	A	A	AI	2	2a
AI	1 CF patient; United Kingdom (Edinburgh)	C1911	1 067 577, *B. cepacia* (93.3), *P. aureofaciens* (6.7)	A	A	I	A	A	AI	2	2a
M	1 CF patient; France (Reims); sputum	1.45	1 447 577, *B. cepacia* (99.9)	A	A	I	A	A	A	2	2b
C	1 CF patient; France (Nancy); sputum	1.4	5 467 577, *B. cepacia* (99.9)	A	A	I	A	A	A	2	2b
AZ	1 CF patient; France (Dijon); sputum	2.60	1 047 577, *B. cepacia* (68.5), *P. aureofaciens* (28.7), *P. fluorescens* (2.8)	A	A	I	A	A	A	2	2b
BH	1 CF patient; France (Roscoff); sputum	3.30	1 067 577, *B. cepacia* (93.3), *P. aureofaciens* (6.7)	A	A	I	A	A	A	2	2b
BL	1 CF patient; France (Paris); sputum	3.78	1 047 577, *B. cepacia* (68.5), *P. aureofaciens* (28.7), *P. fluorescens* (2.8)	A	A	I	A	A	A	2	2b
AT	1 CF patient; Denmark (Copenhagen)	A45	1 467 573, *B. cepacia* (99.9)	A	A	I	A	A	A	2	2b
AE	1 CF patient; United Kingdom (Edinburgh)	C1409	1 467 573, *B. cepacia* (99.9)	A	A	I	A	A	A	2	2b
Q	1 CF patient; Canada (Vancouver, British Columbia)	FC102	5 567 573, unidentified	A	A	I	A	A	A	2	2b
Y	1 CF patient; France (Paris); sputum	1.78	1 047 573, unidentified	A	A	I	A	A	I	2	2c
H	1 CF patient; France (Giens); sputum	1.15	1 467 553, unidentified[b]	A	A	I	A	A	I	2	2c
BC	1 CF patient; France (Suresnes); sputum	2.65	5 467 573, unidentified[b]	A	A	I	A	A	I	2	2c
AR	1 CF patient; Denmark (Copenhagen)	A43	5 067 573, unidentified[b]	A	A	I	A	A	I	2	2c
AS	1 CF patient; Denmark (Copenhagen)	A44	1 467 573, *B. cepacia* (99.9)	A	A	I	A	A	I	2	2c
AU	1 CF patient; Denmark (Copenhagen)	A46	5 047 577, *B. cepacia* (99.9)	A	A	I	A	A	I	2	2c
L	5 CF patients; France (Clermont-Ferrand); sputum	1.36	0 467 573, *B. cepacia* (99.9)	A	A	A	A	AH	AH	1	1a
B	7 CF patients; Canada (Vancouver, British Columbia; ET12 British-Canadian epidemic clone)	C5424 (16)	0 477 573, *B. cepacia* (99.9)	A	A	A	A	A	A	1	1b
D	8 CF patients; France (Nancy); sputum	1.6	1 477 577, *B. cepacia* (99.9)	A	A	A	A	A	A	1	1b
A	26 CF patients; France (Brest, 3; Roscoff, 10; Nancy, 7; Paris, 1; Pessac, 1; Dax, 2; Giens, 1; Dijon, 1); sputum; 1 CF patient; Canada (Vancouver, British Columbia); 17 non-CF patients; France (Bordeaux); blood culture, urine, respiratory specimens	1.1	1 467 573, *B. cepacia* (99.9)	A	A	A	A	A	A	1	1b
BP	12 non-CF patients; Senegal (Dakar); blood culture	DK1	0 467 577, *B. cepacia* (99.9)	A	A	A	A	A	A	1	1b
E	2 CF patients; France (Toulouse); sputum	1.24	0 077 577, *B. cepacia* (85.7), *P. aureofaciens* (14.2)	A	A	A	A	A	A	1	1b
O	1 CF patient; France (Roscoff); sputum	1.51	0 456 557, unidentified	A	A	A	A	A	A	1	1b
AW	1 CF patient; Canada (Cleveland); sputum	K128	0 477 573, *B. cepacia* (99.9)	A	A	A	A	A	A	1	1b
AH	1 CF patient; United Kingdom (Edinburgh)	A552	0 477 573, *B. cepacia* (99.9)	A	A	A	A	A	A	1	1b
V	1 CF patient; France (Giens); sputum	1.58	0 077 577, *B. cepacia* (85.7), *P. aureofaciens* (14.2)	A	A	A	A	A	A	1	1b
BA	1 CF patient; France (Giens); sputum	2.64	1 067 573, *B. cepacia* (81.0), *P. aureofaciens* (19)	A	A	A	A	A	A	1	1b
CA	1 non-CF patient; France (Toulouse); tracheal aspirate, urine	N25	0 477 577, *B. cepacia* (99.9)	A	A	A	A	A	A	1	1b
CB	1 CF patient; France (Dijon); sputum	3.2	1 047 577, *B. cepacia* (68.5), *P. aureofaciens* (14.2), *P. fluorescens* (2.8)	A	A	A	A	A	A	1	1b
BK	1 non-CF patient; France (Toulouse); blood culture	N2	5 077 577, *B. cepacia* (99.9)	A	A	A	A	A	A	1	1b
AF	1 CF patient; United Kingdom (Edinburgh)	A599	0 467 573, *B. cepacia* (99.9)	A	A	A	A	A	AH	1	1c
CC	1 CF patient; France (Giens); sputum	3.5	0 057 577, *B. cepacia* (50.3), *P. aureofaciens* (48.9)	A	A	A	A	H	H	1	1d
AG	1 CF patient; United Kingdom (Edinburgh)	A548	0 467 573, *B. cepacia* (99.9)	A	A	A	A	H	H	1	1d
R	1 CF patient; France (Nantes); sputum	2.44	0 456 577, *B. cepacia* (99.3)	A	B	A	B	H	H	3	
AC	1 CF patient; Canada (Calgary, Alberta); sputum	K130	4 456 573, unidentified[b]	A	B	A	B	H	H	3	
U	2 CF patients; France (Suresnes, 1 patient; Toulouse, 1 patient); sputum	2.10	1 067 577, *B. cepacia* (93.3), *P. aureofaciens* (6.7)	A	A	J	A	A	A	4	
BE	1 CF patient; France (Dax); sputum	2.75	0 067 553, unidentified	B	B	B	B	A	A	5	5a
BG	1 CF patient; France (Paris); sputum	2.72	1 057 577, *P. aureofaciens* (59.9), *B. cepacia* (39.5)	B	B	F	B	A	A	6	
AP	1 CF patient; Denmark (Copenhagen)	A41	5 067 573, unidentified[b]	A	A	C	A	A	AI	14	
P	2 CF patients; Canada (Vancouver, British Columbia)	C5876	0 433 077, unidentified	A	A	A	G	A	A	15	
AX	1 CF patient; France (Toulouse); sputum	2.57	0 057 577, *B. cepacia* (50.3), *P. aureofaciens* (48.9)	B	B	C	B	H	H	16	

[a] If known.

[b] Strain would be identified as *B. cepacia* (% identification of >99%) if oxidase positive.

[c] The groups are defined on the basis of the RFLP profiles obtained with *Alu*I, *Cfo*I, *Dde*I, and *Msp*I, and the subgroups are further defined with *Nci*I and *Bss*KI restriction results.
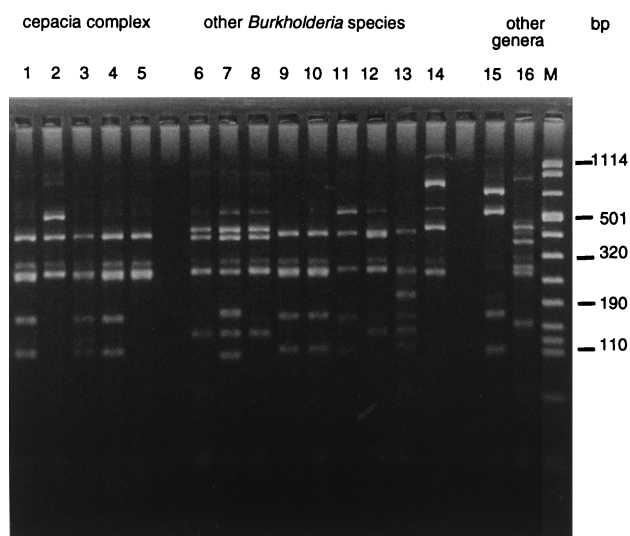
FIG. 1. *Dde*I restriction patterns (type and reference strains). Lanes: 1 to 5 (cepacia complex strains), *B. cepacia* genomovar I ATCC 25416[T], *B. cepacia* genomovar II (*B. multivorans*) LMG 13010[T], *B. cepacia* genomovar III LMG 12615, *B. cepacia* genomovar IV LMG 14294, and *B. vietnamiensis* TVV75[T], respectively; 6 to 14 (other *Burkholderia* species), *B. gladioli* pv. gladioli CFBP 2427[T], *B. gladioli* pv. alliicola CFBP 2422[T], *B. cocovenenans* LMG 11626[T], *B. pyrrocinia* LMG 14191[T], *B. caryophilli* LMG 2155[T], *B. andropogonis* LMG 2129[T], *B. plantarii* LMG 9035[T], *B. glumae* CFBP 2430[T], and *B. glathei* LMG 14190[T], respectively; 15 and 16 (other genera), *R. pickettii* ATCC 27511[T] and *Stenotrophomonas maltophilia* ATCC 13637[T], respectively; M, molecular weight marker VIII (Boehringer Mannheim).

profile with *Dde*I as for the *B. multivorans* type strain as shown in Table 1 and is further divided by *Bss*KI into three RFLP subgroups (2a, 2b, and 2c). Subgroups 2b and 2c exhibit a plain *Bss*KI pattern (A and I, respectively), whereas subgroup 2a exhibits the composite AI pattern like the *B. multivorans* type strain. This group comprises CF strains only, recovered from French, British, Danish, and Canadian patients, and includes two of the five clones proven to be highly transmissible in French CF centers (types F and X). (ii) Group 1 (17 of 51 strains [33%]) is characterized by an "A" profile with *Dde*I as for *B. cepacia* genomovar I and III representative strains and is further divided by *Nci*I and *Bss*KI, again according to whether the profile is plain (A and H) or composite (AH), into four RFLP subgroups (1a, 1b, 1c, and 1d), one of which (1b) is largely predominant. This group comprises CF isolates recovered from French, British, and Canadian patients and nosocomial isolates from France and Senegal. Five epidemic clones, involved in CF (the British-Canadian highly transmissible C5424 strain [7, 16] and the French types L, D, and A) and/or in nosocomial infections (types A and BP), belong to this group. Groups 3, 4, 5, and 6 could be respectively related to the *B. cepacia* genomovar IV reference strain or to *B. pyrrocinia*[T], *B. vietnamiensis*[T], *B. gladioli* pathovar gladioli[T], and *B. gladioli* pathovar alliicola[T] RFLP profiles and comprise a low number of CF strains (one to two strains). The three last groups (14–16) could not be clustered with any of the type or reference strains tested and comprise one CF strain each.

Biotype 100 assimilation tests. One of the 18 reference strains (*B. glathei* LMG 14190[T]) and 5 of the 35 clinical isolates tested did not grow in Biotype 100 strips (typability, 82%). Thus, auxanographic results were available for 17 reference strains and 30 clinical isolates belonging to 8 of the 9 RFLP groups defined as described above. The main discriminating features are presented in Table 3. Strains belonging to RFLP group 2 (17 strains tested) were characterized by the constant absence of growth on sucrose which, in contrast, was assimilated by all the strains belonging to RFLP group 1 (12 strains tested). Sucrose assimilation was found to be generally absent for the type strains of other *Burkholderia* species, except for the *B. vietnamiensis*, *B. caryophilli*, and *B. pyrrocinia* type strains. Assimilation of DL-lactate was also found to be absent in most of the strains (15 of 17) belonging to RFLP group 2, whereas it did occur with most of the other strains tested (28 of 30). Within RFLP group 1, the results of some auxanographic tests, such as the benzoate, putrescine, and histamine tests, were found to be different for reference strains representative of genomovars I and III and heterogeneous among clinical strains. Numerous auxanographic features observed for the *B. vietnamiensis* type strain were markedly different from those observed for the RFLP-related clinical strain. In the same way, heterogeneous results were obtained within RFLP group 3.

DISCUSSION

The accurate identification of *B. cepacia* is critical with regard to the medical and sociological consequences of the diagnosis for patients with CF. Nevertheless, the levels of pathogenicity and transmissibility within colonizing *B. cepacia* strains have been shown to be highly variable, and biologic prognostic markers are not available yet. On the other hand, there is increasing evidence (8, 25) that *B. cepacia* is a complex of closely related species rather than a single species. Moreover, another species of the genus *Burkholderia*, *B. gladioli*, is also involved in CF, but its pathogenic potential is still being discussed (4, 23). Thus, the unambiguous differentiation of *Burkholderia* species is undeniably the first obligatory step of any clinicobiological analysis of infections due to these organisms.

Phenotypic identification techniques are generally considered to be insufficiently reliable for this purpose, an observation which was confirmed by the present study. Fifty-one presumed clinical *B. cepacia* isolates were analyzed with the API 20NE numerical identification system, which is widely used in clinical microbiology laboratories, which was completed by use of the oxidase test. The following difficulties were encountered: (i) the poor detection of oxidase; (ii) the absence of *Burkholderia* species other than *B. cepacia* in the database; (iii) the inability to differentiate *B. cepacia* from *P. aureofaciens*, as previously reported by Kiska et al. (12) with the API Rapid NFT system; and (iv) the high number of unidentified strains (11 of 51 isolates [21%]). However, misidentifications at the genus level were rare (1 of 51 isolates [2%]).

Genomic analysis might provide more reliable tools for identification. So far, the reference method is the evaluation of DNA-DNA homologies between strains, which requires high technical skill and is time-consuming and thus not appropriate for routine diagnosis. On the other hand, the composition of 16S rDNA sequences, which are generally species specific, can be easily approached by analyzing the endonuclease restriction profiles of the previously amplified 16S rDNA gene. Furthermore, the selection of discriminating enzymes is facilitated by the fact that 16S rDNA sequences are available for many bacterial species. This method has been previously applied by Laguerre et al. (14) to the differentiation of fluorescent *Pseudomonas* species, and its use in the identification of *Burkholderia* species was assessed in the present study.

Of the 13 enzymes used by Laguerre et al., 6 (*Alu*I, *Cfo*I, *Dde*I, *Msp*I, *Nci*I, and *Bss*KI) were used in the present study; four of these (*Alu*I, *Cfo*I, *Dde*I, and *Msp*I) proved to be the more useful in species classification. However, this method did not allow the differentiation of genomovar I and III represen-

TABLE 3. Main discriminant auxanographic features based on the study of 17 reference strains and 30 clinical isolates

Strain tested[a]	No. of strains tested	Substrate[b]										
		Sucrose	L(−)-Arabitol	D(−)-Tartrate	Ben-zoate	5-Keto-D-gluconate	Genti-sate	Putre-scine	DL-Lactate	5-Amino-valerate	Trypt-amine	Histamine
RFLP group 2												
B. multivorans[T]	1	−	+	−	+	−	+	+	−	+	+	+
Clinical strains	16	−	15/16	1/16	15/16	4/16	+	10/16	2/16	13/16	11/16	12/16
RFLP group 1												
Reference strains of *B. cepacia* gv I	3	+	+	−	+	+	+	+	+	+	2/3	+
Reference strain of *B. cepacia* gv III	1	+	+	−	−	+	+	−	+	+	+	−
Clinical strains	8	+	+	−	5/8	+	5/8	4/8	7/8	5/8	3/8	1/8
RFLP group 3												
Reference strain of *B. cepacia* gv IV	1	−	+	−	+	+	+	+	+	+	−	+
B. pyrrocinia[T]	1	+	+	−	+	+	−	+	+	+	+	+
Clinical strain	1	−	+	−	+	+	+	+	+	+	+	+
RFLP group 4												
B. vietnamiensis[T]	1	+	−	−	+	−	−	+	+	+	−	+
Clinical strain	1	−	+	−	+	−	+	−	−	−	−	−
RFLP group 5												
B. gladioli pathovar gladioli[T]	1	−	−	+	+	+	−	−	+	−	−	−
B. cocovenenans[T]	1	−	−	+	+	+	−	−	+	−	−	−
Clinical strain	1	−	−	+	−	−	−	−	+	−	−	−
RFLP group 6												
B. gladioli pathovar alliicola[T]	1	−	−	+	+	−	−	−	+	−	−	−
Clinical strain	1	−	−	−	−	+	−	−	+	−	−	−
RFLP group 15, clinical strain	1	+	+	−	+	+	+	+	+	−	−	−
RFLP group 16, clinical strain	1	+	−	+	−	−	+	−	+	+	−	−
RFLP group 7, *B. plantarii*[T]	1	−	−	+	−	−	−	−	+	−	−	−
RFLP group 8, *B. caryophilli*[T]	1	+	−	−	−	+	−	−	+	−	−	−
RFLP group 9, *B. andropogonis*[T]	1	−	−	−	−	−	−	−	+	−	−	−
RFLP group 10, *B. glumae*[T]	1	−	−	−	+	−	−	−	+	−	+	−
RFLP group 12, *R. pickettii*[T]	1	−	−	−	−	−	−	−	+	−	−	+
RFLP group 13, *S. maltophilia*[T]	1	+	−	−	−	−	−	−	+	−	−	−

[a] gv, genomovar; [T], type strain. The groups are defined on the basis of the RFLP profiles obtained with *Alu*I, *Cfo*I, *Dde*I, and *Msp*I.
[b] +, all strains tested positive; −, all strains tested negative. Ratios give the number of positive strains over the total number of strains tested.

tative strains, in contrast to the DNA binding values reported by Vandamme et al. (25) between these strains (maximum, 45%). Further studies are required in order to search for more discriminating enzymes; nevertheless, if the low DNA-binding value is due to the different size of the genome, which was shown to be highly variable among *B. cepacia* strains (15), this quest might be unsuccessful. On the other hand, the *Dde*I pattern allowed the differentiation of *B. multivorans*[T] (geno-movar II) and *B. vietnamiensis*[T] from the representative strains of genomovars I and III. Lastly, the 16S rDNA RFLP profile of the *B. cepacia* genomovar IV reference strain tested was clearly different from those obtained with the other genomovars but was indistinguishable from the genotype of the type strain of *B. pyrrocinia* (formerly *Pseudomonas pyrrocinia*). This provides an additional argument for the inclusion of the species *pyrrocinia* into the genus *Burkholderia* (25, 26). The relationship between the two species requires further investigation, since DNA hybridization results between these strains were not published. Reference strains belonging to other *Burkholderia* species, i.e., *B. cocovenenans*, *B. gladioli* (pathovar gladioli and pathovar alliicola), *B. plantarii*, *B. caryophilli*, *B. andropogonis*, *B. glumae*, and *B. glathei*, were all differentiated. It should be observed that the profiles of *B. gladioli* pathovar gladioli and of *B. cocovenenans* are very close, differing only by the *Bss*KI restriction profile; the high level of similarity between these species has been previously reported (26). The easy differentiation of *B. gladioli* from strains of the cepacia complex is particularly interesting since phenotypic features described as discriminating between *B. gladioli* and *B. cepacia*, i.e., negative oxidase

and lysine decarboxylase (LDC), the absence of lactose and maltose fermentation, and aminoglycoside susceptibility, may be equivocal (23).

In a second step, the PCR-RFLP method has been applied to a large variety of presumed *B. cepacia* isolates recovered from CF or nosocomial infections that are either epidemic or sporadic and are from different countries. The previous typing of all the isolates by PCR ribotyping allowed a selection of genetically unrelated strains to assess the diversity within this bacterial group. Nine RFLP groups were delineated among these clinical strains on the basis of the restriction patterns obtained with four of the six restriction enzymes (*Alu*I, *Cfo*I, *Dde*I, and *Msp*I). However, further variations could be defined within groups 2 and 1, by *Nci*I and/or *Bss*KI analysis, with plain (A, H, and I) and composite (AH and AI) profiles. The existence of composite profiles may be attributed to variations among the different operons, since *B. cepacia* was shown to harbor at least six rDNA operons (3, 15, 19). Whether the subgroups determined by *Nci*I and/or *Bss*KI reflect intraspecific or interspecific variations is unclear. Since similar variations were observed among the three type and reference strains belonging to genomovar I, the analysis of a large number of reference strains of each species is required to evaluate intraspecific heterogeneity with regard to *Nci*I and *Bss*KI restriction profiles. In the present study, CF isolates were mainly distributed in RFLP groups 2 and 1 which, respectively, include the *B. multivorans* type strain and *B. cepacia* reference strains classified by Vandamme et al. in the genomovars I and III, whereas the distribution of the few nosocomial isolates studied

herein was restricted to RFLP group 1. The easy differentiation of genomovars I and III would be interesting, since Vandamme et al. showed than genomovar III strains were mainly involved in CF, whereas genomovar I strains were mainly recovered from natural and hospital environments and from non-CF patients (25). None of the phenotypic features described by Vandamme et al. (25) appears to be strictly specific to genomovar I or III, the most constant one being the absence of growth of genomovar I strains at 42°C. Whereas most of the highly transmissible strains and all the isolates associated with acute clinical decline (8, 25) were previously reported to belong to genomovar III, our data show that among the five highly transmissible clones in French CF centers, three (A, L, and D) actually belong to the genomovar I/III-related RFLP group, but two (F and X), of which one (F) has been associated with fatal septicemias, belong to the *B. multivorans*-related RFLP group. The phenotypic differentiation of *B. multivorans* proposed by Vandamme et al. (25) is based upon a low number of characters and is not really clear-cut. One of the interesting characters could be LDC determination, reported to be negative in the description of the species *B. multivorans* but not documented for the other genomovars; LDC has been previously reported to be positive in 80% of *B. cepacia* strains (18). In our study, phenotypic analysis was restricted to auxanographic data, and the sole distinctive feature was the absence of growth of *B. multivorans*-related strains on sucrose. The discriminating value of this character requires confirmation on a higher number of strains.

Three other species and/or genomovars are also involved, though rarely, in CF patients: *B. gladioli*, *B. vietnamiensis*, and *B. cepacia* genomovar IV (4, 6, 25). The accurate identification of these species is essential in order to evaluate their pathogenic potential in CF and thus for guiding the segregation and treatment of patients who are infected with these bacteria. In our study, five isolates could be related to these species, though the relation between *B. cepacia* genomovar IV and *B. pyrrocinia*, which share the same RFLP pattern, requires further investigation. Finally, three isolates exhibiting unique RFLP profiles (14–16) could not be related to any representative of the *Burkholderia* species tested, suggesting the involvement of as-yet-uncharacterized species in CF.

In conclusion, the PCR-RFLP method described here appears to be an efficient identification tool for *Burkholderia* species. Its taxonomic value has to be validated by the analysis of a larger number of reference strains and above all by DNA-DNA hybridization studies. The use of additional endonucleases might also improve its discriminatory power within the *B. cepacia* complex. If these preliminary results are confirmed, the newly individualized *B. multivorans* species should be considered to play a significant part in CF and to include transmissible and virulent clones to the same extent as the *B. cepacia* genomovar III strains.

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